

APPENDIX 1

Non-Obese Diabetic (NOD) Mouse BAC Library

The Wellcome Trust Sanger Institute (Cambridge, United Kingdom) recently released to the scientific community a Non-Obese Diabetic (NOD) mouse BAC library containing 240,000 clones obtained from the Diabetes and Inflammation Laboratory (Cambridge, United Kingdom).

The NOD mouse, which spontaneously develops type 1 diabetes, is a valuable animal model that is used extensively in research exploring the etiology, prevention, and treatment of this disease. It is a vital research tool for testing promising prevention and treatment strategies at the preclinical level.

The Sanger Institute sequenced the complete NOD BAC library and used this resource to complete a physical map of the BAC clones. As a next step, they plan to sequence the 200,000 clones from the Pieter de Jong library (Children's Hospital, Oakland CA). These sequences will be aligned with those from the most recent version of the normal C57B1/6 (B6) mouse strain (a non-diabetic mouse strain) in an effort to identify single nucleotide differences between NOD mouse clone end sequences and the B6 mouse genome. Investigators at the Sanger Institute will use this sequencing information to identify and map candidate genes. Such information will guide efforts to isolate genes that contribute to the development of type 1 diabetes in humans.

This research was conducted as part of the Immune Tolerance Network, which is jointly funded by the National Institute of Allergy and Infectious Diseases, the National Institute of Diabetes, Digestive, and Kidney Disorders (both part of the National Institutes of Health) and the Juvenile Diabetes Research Foundation.

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APPENDIX II

Development of new strategies to prevent type 1 diabetes: the role of animal models

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Type 1 diabetes is an immune-mediated disease typically preceded by a long preclinical stage during which a growing number of islet-cell-specific autoantibodies appear in the serum. Although antigen-specific T lymphocytes and cytokines rather than these autoantibodies are the likely executors of β -cell-destruction, these autoantibodies reflect the existence of autoimmunity that targets islet β -cells. Abrogation of this autoimmunity during the pre-clinical stage would be the key to the prevention of type 1 diabetes. However, the quest of protecting islet-cells from the immune attack requires detailed knowledge of mechanisms that control islet-inflammation and β -cell-destruction, and of mechanisms that control immune tolerance to peripheral self-antigens in general. This knowledge can only be obtained through further innovative research in experimental animal models. In this review, we will first examine how research in non-obese diabetic mice has already led to promising new strategies of diabetes prevention now being tested in human clinical trials. Thereafter, we will discuss how recent advances in understanding the mechanisms that control immune response to peripheral self-antigens such as β -cell antigens may help to develop even more selective and effective strategies to prevent diabetes in the future.

Keywords: antigen-presentation; dendritic cells; disease prevention; immunological tolerance; non-obese diabetic mice; T-lymphocytes; transgenic animal models; type 1 diabetes

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Introduction

Type 1 diabetes (T1DM) is the clinical manifestation of the loss of insulin production in the endocrine pancreas. This is caused by immune-mediated destruction of islet β -cells. T1DM is believed to be an autoimmune disease, based on several lines of evidence (1–9). Although an infective (e.g., viral) or other environmental (e.g., dietary) agent may well be involved in the initiation of the immune attack towards islet β -cells (10,11), it is clear that the (auto)immune attack itself is a key element in disease pathogenesis (12).

Immune responses against various islet-antigens appear even years before the clinical manifestation of T1DM, and precede the phase when sensitive metabolic tests first reveal attenuation in the function of β -cells (13). A number of islet-antigens are targeted by the immune system in T1DM, and cellular and/or humoral immune responses are detected against insulin, glutamic acid decarboxylase-65 (GAD 65), tyrosine phosphatase IA-2 and heat shock protein 60 (hsp 60) (14–16). Studies on pancreas samples obtained shortly after onset of clinical disease (as biopsies or as autopsy material following fatal ketoacidosis) have revealed the existence of cellular infiltrates in islets consisting of lymphocytes and antigen-presenting cells (APC), reflecting the immune attack and selective destruction of insulin-producing β -cells (4–6). Although studies in humans have been essential in the characterization of the disease process and its target antigens, development of novel therapeutic strategies requires studies also in experimental animal models.

The non-obese diabetic (NOD) mouse is the most widely used animal model of T1DM (17). This inbred mouse strain is unique in that it spontaneously develops autoimmune diabetes at high incidence. Although NOD mice harbour certain unique defects in their immune system (such as lack of the murine homolog of human leukocyte antigen HLA-DR (18) and complement component C5 (19)) and have a

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Abbreviations and acronyms

APC	antigen-presenting cell
CTLA-4	cytotoxic T-lymphocyte antigen-4
DC	dendritic cells
GAD65	glutamic acid decarboxylase-65
GM-CSF	granulocyte-macrophage colony-stimulating factor
HLA	human leukocyte antigen
IA-2	islet-associated-2 (tyrosine phosphatase, an autoantigen)
IL	interleukin
IFN	interferon
hsp 60	heat shock protein 60
LCMV	lymphocytic choriomeningitis virus
MHC	major histocompatibility complex
NKT	natural killer cell-like T cell
NOD	non-obese diabetic mouse
OT-I T cell	OVA-specific CD8 T cell
OVA	ovalbumin
PD-L1	programmed death ligand 1
RANK	receptor for activation of NF κ B
RIP	rat insulin promoter
TGF	transforming growth factor
T1DM	type 1 diabetes mellitus
TCR	T cell receptor
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
TRANCE	TNF-related activation induced cytokine
Treg	regulatory T cell

Key messages

- Type 1 diabetes (T1DM) is the clinical manifestation of immune-mediated destruction of insulin-producing β -cells which cannot be prevented yet.
- Most of the current clinical trials aiming at T1DM prevention are based on strategies developed in animal models.
- Research with these animal models has recently generated essential information regarding the cellular and molecular mechanisms regulating immune response to β -cells that can be used in the development of novel prevention strategies.

strong gender bias in diabetes incidence towards female preponderance (20), many of the important determinants are strikingly similar, including homologous susceptibility genes, major autoantigens, existence of insulinitis and the ability of bone-marrow cells to transmit diabetes between individuals (21, 22).

In this review, we will first describe how research in non-obese diabetic mice has led to the discovery of new strategies of diabetes prevention that are now in human clinical trials. Thereafter, we will focus on work in transgenic animal models of islet-autoimmunity and discuss how immune tolerance towards sequestered self-antigens such as β -cell antigens is maintained, and how the tolerant state can change into a pathogenic immune response. Emphasis will be given to work elucidating the cellular and molecular basis of this balance and on therapeutic approaches that aim at posing this balance away from pathological autoimmunity.

The NOD mouse – a platform for testing existing immunomodulatory agents in the prevention of spontaneous autoimmune diabetes

Application of modern methods in molecular and cell biology to research on the mammalian immune system

has allowed numerous receptor-ligand pairs and intracellular signalling pathways that regulate the function of lymphocytes and antigen-presenting cells to be identified. Through this research, an increasing number of antibodies and other reagents have become available for selective blockade or mimicking of the function of a specified molecule with a regulatory function. The NOD mouse is ideal for testing the effect on diabetes incidence of such agents. Thus, it forms a platform for identification and validation of potential drug targets and drugs in diabetes prevention.

In fact, surprisingly many different manoeuvres (over a hundred or so) have been reported to delay or diminish diabetes incidence in NOD mice (17). Many of these lack direct relevance for prevention of T1DM in humans simply because of their experimental nature. However, several potentially relevant approaches have been identified (Table 1 and references therein). As will be discussed later, some of the earliest human trials based on these approaches have now given negative outcomes that could reflect differences between T1DM in humans and the corresponding syndrome in NOD mice. However, results of other trials are more consistent with those obtained in NOD mice and support the idea that the NOD model can be used as a platform for testing various prevention protocols. Protocols applied in the NOD model have been based firstly on blocking the activation or function of T-lymphocytes or their subtypes and hence, of pathogenic effector cells, or their migration into pancreatic islets. Secondly, they have been based on administration of autoantigens in forms and schedules that are anticipated to enhance regulation of specific immune responses. Thirdly, numerous antigen-non-specific substances with immunomodulatory effects, or possible protective effect on β -cells have been applied with often favourable outcomes.

It is conceivable that targeting a subtype of T-lymphocytes or a costimulatory pathway will affect the immune system also on a general level and induce various side effects. Therefore, there has long been a quest to develop strategies that selectively target the immune effector cells or locally inhibit tissue destruction. Accordingly, autoantigens (pro)insulin, GAD65 and hsp-60 or their peptides have been administered without immune adjuvants and/or *via* tolerogenic routes, especially intravenously or *via* mucosal surfaces (orally, intranasally or inhaled as aerosol). Results of these experiments have been promising (Table 1 and references therein) and have already led to human clinical trials, as detailed below. Unfortunately, initial results of these trials have been less encouraging (see below). According to animal experiments, oral and intranasal administration of antigen can also lead to induction of cytotoxic T-cell immunity (53–55) that may counteract its tolerance-promoting effects. This might need more attention in current trials where autoantigens are administered *via* mucosal routes. In animal models, a promising new strategy to induce regulatory immunity and protection from autoimmune diseases including diabetes is

genetic (that is, DNA) vaccination with plasmids that encode an autoantigen (56). This strategy will be discussed later in this review.

Current clinical trials based on strategies developed in NOD mice

Strategies employed currently in clinical trials to prevent T1DM include the use of autoantigens in a tolerance-promoting form, targeting T-lymphocytes with antibody, protecting islet β -cells from the action of inflammatory cytokines and non-specific stimulation of regulatory cell types. Most of the ongoing trials, which are summarized in Table 2, are based on strategies developed in the NOD mouse. We will describe these trials shortly.

Anti-CD3 monoclonal antibody

hOKT3 γ 1[Ala-Ala] is a humanized non-activating anti-CD3 antibody engineered to lack Fc-Receptor binding domains (57). Preclinical studies in the NOD mouse showed that antibody treatment could effec-

Table 1. Examples of protocols that prevent diabetes in NOD mice and have a relevant target mechanism.

Protocol	Mechanism of action	Antigen specificity	Reference
Treatment with an antibody against:			
CD3	Depletion or inactivation of islet-infiltrating T cells/induction of regulatory immunity	no	(23, 24)
CD4/CD8	Depletion/inactivation of CD4 or CD8 T cells	no	(25)
TCR	Depletion/inactivation of T cells	no	(26)
CD86(B7.2)	Interference with T cell activation	no	(27)
CD45RB	Interference with T cell activation	no	(28)
CD40L	Interference with T cell activation	no	(29)
VLA-4 (α 4-integrin)	Prevention of lymphocyte accumulation in islets and/or activation of diabetogenic T cells	no	(30, 31)
MAcCAM-1	Prevention of lymphocyte accumulation in islets and/or activation of diabetogenic T cells	no	(32)
ICAM-1/LFA-1	Prevention of lymphocyte accumulation in islets and/or activation of diabetogenic T cells	no	(33)
Treatment with:			
adjuvant (IFA)	immune deviation	no	(34)
vitamin D3 deriv.	immune deviation	no	(35)
α Gal-ceramide	activation of regulatory NK T cells	no	(36)
interleukin 4	immune deviation/regulatory cells	no	(37)
interleukin-10	immune deviation/regulatory cells	no	(38, 39)
nicotinamide	protection of target β -cells against NO	no	(40)
subcutaneous insulin	Inactivation of pathogenic T cells	yes	(41, 42)
oral, intranasal or aerosol insulin	Induction of regulatory T cells/inactivation of pathogenic T cells	yes	(43–46)
insulin-encoding DNA-plasmid	Induction of regulatory T cells/inactivation of pathogenic T cells	yes	(47)
intranasal GAD peptides	Induction of regulatory T cells/inactivation of pathogenic T cells	yes	(48)
GAD-encoding DNA-plasmid	Induction of regulatory T cells/inactivation of pathogenic T cells	yes	(49, 50)
intrathymic GAD	Deletion of GAD-specific T cells from the pool of circulating mature T cells	yes	(51)
HSP 60	Inactivation of pathogenic T cells	yes	(52)
p277 of HSP 60	Inactivation of pathogenic T cells	yes	(52)

Table 2. Currently ongoing clinical trials to prevent diabetes.

Trial	Principle/hypothesis	Evidence from NOD mice	Status	Results ^a
hOKT-3 γ 1	Inactivation of T cells. Induction of regulatory immunity	yes	Phase I-II	+
p277 of HSP-60	Induction of tolerance towards one autoantigen	yes	Phase I	+
insulin i.v. and s.c. ^b	Induction of tolerance towards one autoantigen	yes	Phase II	–
insulin s.c. in adjuvant (IFA) ^c	Induction of tolerance towards one autoantigen	yes	Phase II	N.A.
insulin via oral route	Induction of tolerance towards one autoantigen	yes	Phase II	N.A.
insulin via intranasal route	Induction of tolerance towards one autoantigen	yes	Phase III	N.A.
GAD 65 s.c.	Induction of tolerance towards one autoantigen	yes	Phase II	N.A.
nicotinamide via oral route	Protection of islet β -cells	yes	Phase III	–
IFN- α	Modulation of immune response	yes	Phase II	N.A.
Lactobacillus via oral route	Stimulation of regulatory NK-T cells	yes	Phase II	N.A.
Ap ^d of insulin B-chain	Induction of tolerance to insulin	yes	Phase II	N.A.
Vitamin D3 derivative	Modulation of immune response	yes	Phase II	N.A.
Exclusion of bovine proteins from diet in infancy	Avoidance of early immune response towards putative 'mimics' of autoantigens and/or sensitization of immature gut to foreign proteins	not directly	Phase III	N.A.

^a Results: + positive, – negative results; N.A. results not available yet; ^b i.v. = intravenous administration; s.c. subcutaneous administration; ^c IFA = incomplete Freund's adjuvant; ^d ApI = altered peptide ligand.

tively 'cure' diabetic mice, restoring normoglycemia (24) or prevent disease development in pre-diabetic mice (58, 59). The antibody appears to bind to all CD3 expressing T-cells resulting in partial T cell receptor (TCR) signalling, which then has different outcomes depending on the cell type triggered. The overall outcome being killing or anergy induction in Th1 type cells (producing interleukin-2 or interferon- γ) and stimulation of Th2 type cells (cells producing interleukin-4 or interleukin-10) (60, 61). In an intervention trial, a 14 day course of intravenous hOKT3- γ 1[Ala-Ala] was used in recent onset (within 6 weeks) T1DM patients and followed for one year. Treatment resulted in sustained or improved C-peptide responses in 9 out of 12 treated patients compared with a sustained response in only 2 out of 12 control patients. Treated patients also needed significantly less insulin over the year following diagnosis. A transient depletion (36%) of lymphocytes followed antibody treatment, which later returned to normal levels. Responding patients had a decreased ratio of CD4:CD8 T cells after repopulation. Mild side effects included anti-idiotypic antibodies, mild fever and an eczematous dermatitis-like rash. This trial is now being expanded to a multi-centre phase II trial involving about 80 patients after these promising results. Further details are available at <http://www.immunetolerance.org/research/autoimmune/trials/herold1.html>

Hsp-60 peptide p277

One of the many self antigens, which are reacted against in T1DM, is heat shock protein 60. NOD mice contain autoreactive T cells specific for peptide 277 derived from this protein and immunisation of adult NOD mice with this peptide could prevent and

occasionally revert diabetes (52). The mechanism of action is believed to be stimulation of Th2 type hsp60-reactive T cells resulting in a change in the cytokine milieu away from inflammatory cytokines (62). DiaPep277 is the human form of this peptide, which has been modified at two residues to increase stability *in vivo* (63). In a randomised double blind phase II trial DiaPep277 was injected subcutaneously in mannitol and vegetable oil at 0, 1 and 6 months following entry into the trial (63). Adult male patients were on average 12–15 weeks post-diagnosis and were followed for only 10 months. At the end of the study mean C-peptide levels had been maintained in treated patients whereas they had fallen in controls. Insulin requirements were also significantly lower at 10 months. Further phase II and phase III trials are now beginning based on these suggestive results.

Subcutaneous insulin therapy

Insulin has long been viewed as a primary target for tolerisation therapy in T1DM. It was shown that incidence of diabetes was significantly reduced in NOD mice given a low dose of prophylactic insulin from weaning until 180 days of age (41). Similar findings were also observed in the BioBreed rat, another animal model of T1DM (64). This phenomenon was believed to be due to 'beta-cell rest' in which there was a lower requirement for insulin secretion by the beta cells and less release of islet autoantigens associated with insulin secretion. These findings paved the way to the establishment of the Diabetes prevention trial (DPT-1) in which either subcutaneous or oral insulin was given to high risk relatives of T1DM patients. In the subcutaneous branch of the trial 84,228 relatives were assessed immunologically, metabolically and genetically for risk of disease

development over 5 years (DPT-T1D study group 2002). A group of 169 high risk (>50% over 5 years) relatives then underwent intervention of daily s.c. insulin injections and 170 controls received no treatment. The incidence of diabetes after 5 years was identical in both groups (69 *versus* 70 diabetic subjects). The reason for the negative outcome in the DPT-1 trial compared with the positive results in animal models and a pilot trial in humans (65) is unknown. It may be due to the dosage protocol or the difference in time of intervention. In mice, therapy began at a young age before the development of insulinitis whereas relatives were selected on the basis of showing signs of autoimmunity (presence of islet autoantibodies) already.

Mucosal insulin

The second part of the DPT-1 trial is ongoing and used the 'intermediate' risk (25%–50% risk of developing T1DM over 5 years) relatives of T1DM patients. Patients receive continuous oral insulin or placebo and are being followed for 6 years for diabetes development. This intervention was not predicted to prevent diabetes by 'beta-cell rest' as subcutaneous insulin was but rather through active tolerisation upon antigen uptake at a mucosal surface (66). In NOD mice, an active (transferable), form of tolerance can be induced by feeding mice insulin but it is highly dose-dependent (43, 44). Therefore it is likely that in transferring this therapy to the human situation the dosage protocol will be critical.

A second major trial, the diabetes prediction and prevention project (DIPP), also seeks to tolerise individuals predicted to have a high risk of progression to T1DM by mucosal insulin administration. In this study babies are screened at birth for genetic HLA markers predisposing them to T1DM and then undergo immunological follow up. At risk children are then enrolled in a prevention trial to test intranasal insulin *versus* placebo for its ability to prevent or delay disease onset over three years. Aerosol insulin and intranasal insulin peptide administration have both been shown to be efficacious in prevention of diabetes in the NOD model (45, 46), although intranasal antigen administration has also been shown to induce potent cytotoxic T-cell immunity (67). For further details, see <http://www.utu.fi/research/dipp/engdextx.htm>

Subcutaneous insulin B chain in IFA

Another trial also attempts to induce tolerance to the metabolically inactive B chain of insulin by subcutaneous injection in Incomplete Freund's Adjuvant (IFA). In the NOD mouse, subcutaneous insulin or insulin B-chain given in IFA generated a transferable

suppressive effect (42). In this study patients with insulin autoantibodies receive one injection within one month of diagnosis of T1DM and will be followed for two years. For further details, see <http://www.immunetolerance.org/research/autoimmune/trials/orban1.html>

Subcutaneous GAD65 in alum

An important early autoantigen in diabetes in both the NOD mouse and humans is GAD65 (51). A phase II trial will test the ability of recombinant GAD65 in alum to halt disease progression in recent onset patients. The vaccine called Diamyd will be injected subcutaneously two times four weeks apart in a range of doses. For further details, see <http://www.diamyd.com/docs/research.html>

Nicotinamide

A different approach, which is not antigen specific, attempts to use high doses of the B-vitamin nicotinamide to prevent or delay T1DM onset. Nicotinamide acts on the islet beta cells themselves making them more resistant to autoimmune attack. Nicotinamide is thought to primarily target the enzyme poly(ADP-ribose)polymerase (PARP) which is upregulated early after exposure to nitric oxide or reactive oxygen intermediates. Such exposure causes depletion of intracellular nicotinamide adenine dinucleotide (NAD⁺) and results in death of sensitive cell types such as beta cells (68). Early nicotinamide treatment of NOD mice prevented diabetes partially and reduced insulinitis severity (40). The results of initial clinical trials (69–71) have been controversial, since, while Chase et al. reported no effect of oral administration of nicotinamide, both Mendola and collaborators and Pozzilli et al. observed an increased stimulated C-peptide secretion in postpubertal patients on nicotinamide.

Nicotinamide has also been used in prevention trials with relatives of T1DM patients. One German study of 25 nicotinamide treated and 30 placebo control subjects showed no effect on disease progression (72). However, a larger study in New Zealand involving schoolchildren who had islet-cell autoantibodies obtained results that suggested a protective effect (73). The European nicotinamide diabetes intervention trial (ENDIT) is a larger clinical trial involving 552 high-risk relatives taking daily oral nicotinamide or placebo. For further details and for the recently disclosed negative results of this trial, see <http://www.bris.ac.uk/Depts/DivMed/endit.html>

Interferon-α

One successful strategy to prevent disease in the NOD

mouse involves altering the cytokine milieu to an anti-inflammatory, i.e., interleukin (IL)-4 and IL-10 containing environment. This has been done by injection of these cytokines, transgenic expression or by stimulation of cells which can produce them endogenously. Brod and co-workers have fed NOD mice with interferon- α , which suppressed diabetes and stimulated mitogen induced IL-4, IL-10 and interferon (IFN)- γ production in splenocytes (74). This was then trialled in 10 newly diagnosed T1DM patients resulting in preserved beta-cell function in 8 of these patients after 12 months (75). This study has now been expanded to a larger phase II trial involving 120 newly diagnosed patients taking oral IFN- α daily. It is unclear however what the exact mechanism of this therapy is; as interferon administered orally does not appear to be adsorbed into the bloodstream (76). Additionally, the presence of transgenically expressed IFN- α in the islet beta cells themselves actually precipitates diabetes (77). As such it is possible that interferon- α acts on lymphocytes in the gut, which then circulate to have a wider regulatory effect.

Oral administration of lactobacillus

Another study also attempts to modulate the immune response *via* the gut. Maclaren and co-workers are conducting a trial of oral lactobacillus in recently diagnosed T1DM patients. They propose that this will stimulate natural killer cell-like T (NKT) cell activity as administration of lactobacillus plantarum to children with HIV boosted this cell-subset (78). NKT cells are a regulatory-cell subset which have been reported to be deficient in T1DM patients (79, 80) although a more recent study utilising a more direct tetramer based method of identifying NKT cells refutes this claim (81). In the NOD mouse both transfer of this cell subset or injection of a glycolipid ligand to activate existing NKT cells can prevent diabetes (36, 82, 83). Lactobacillus casei feeding to NOD mice prevents disease (84) and various lactobacilli strains have been shown to be strong stimulators of IL-12 and IL-10 (85). However, strong evidence of lactobacillus stimulation of NKT cells has yet to be demonstrated.

Altered peptide ligand NBI-6024

An interesting antigen specific therapy involves the identification of peptides with similar sequences to immunodominant epitopes, which have modulatory activity. In the NOD mouse peptide 9-23 of the insulin B chain is recognised by a large proportion of pathogenic CD4 T-cells derived from the insulinitic infiltrate (86-88). Alleva and colleagues searched for peptide analogues of B₍₉₋₂₃₎ which could inhibit B₍₉₋₂₃₎ specific T-cell responses and used one of these,

NBI-6024, to test its therapeutic effect in the NOD mouse (89). Therapeutic altered peptide ligands are thought to function as competitive inhibitors of the native peptide by having a high binding affinity for MHC while concurrently engaging the TCR in a non-productive manner (90). They have also been shown to be effective in stimulating regulatory cell subsets, which can transfer protection between animals (91). The altered peptide ligand NBI-6024 is now being used in a large phase II study in which new onset T1DM patients will receive monthly injections of this medication at three different doses for two years.

1,25-Dihydroxy-Vitamin D3

The activated form of vitamin D3, 1,25-Dihydroxy-Vitamin D3, has been shown to act directly on the immune system *via* specific receptors on APCs and activated T cells (92). Its effects are immunosuppressive and include inhibition of IL-2 and IL-12 production and Th1-type responses (93). Importantly 1,25-Dihydroxy-Vitamin D3 has been shown to inhibit dendritic cell maturation, both *in vitro* and *in vivo* (94, 95). Administration of an analogue of 1,25-Dihydroxy-Vitamin D3 to NOD mice significantly reduced the incidence of diabetes (96) and enhanced the number of CD4⁺ CD25⁺ cells of regulatory phenotype in the pancreatic lymph node (97). 1,25-Dihydroxy-Vitamin D3 administered daily for 9 months in recently diagnosed T1DM patients is being trialled in a German study. For further details on this trial see <http://www.roche.com/pages/downloads/science/pdf/rtdcmannh02-3.pdf>

Withdrawal of cow's milk proteins from diet during infancy

According to one hypothesis (not directly derived from findings in NOD mice), exposure of the immature digestive tract in infancy to casein or other proteins in cow's milk formula or soy-based formula has a role in the early pathogenesis of type 1 diabetes. A casein-free diet has been tested in the NOD mouse after weaning and was shown to be effective in preventing diabetes (98). Although contradictory results were achieved from previous studies in humans evaluating whether introduction of formula during infancy is associated with the development of type 1 diabetes later in life (99, 100), a larger phase III clinical trial called TRIGR (Trial to Reduce IDDM in the Genetically at Risk) has been initiated to test if the hypothesis holds true or not. Infants that are determined to have a high risk of developing type 1 diabetes are eligible. After weaning from breastfeeding, they will receive hydrolyzed formula that does not contain intact proteins, or standard cow's milk-based formula. Infants will have at least a two-

month exposure to the study formulae and then will be monitored for up to ten years. For further details, see <http://www.trigr.org/about.html>

Transgenic mouse models in the study of T cell-mediated autoimmunity against pancreatic islets

Investigations in humans with T1DM and studies performed in the NOD mouse model have clearly demonstrated the crucial role of autoreactive CD4 helper and CD8 effector T cells in the pathogenesis of autoimmune diabetes (101). In NOD mice, numerous autoantigens are recognized by these T cells (see above). The relevance of these antigens for disease initiation and progression, and the mechanisms of their processing and presentation, however, are still unclear. Consequently, techniques and tools other than the NOD model had to be developed. The most valuable models were generated by genetically introducing well characterized model antigens into islet- β -cells, under the assumption that such neoantigens will be handled like endogenous islet antigens by the immune system (102). Transgenic neoantigens, however, are often aberrantly expressed in the thymus, resulting in the deletion of endogenous transgene-specific T cells. This problem could be overcome by intravenous injection of specific T cells, which are usually obtained from congenic T cell receptor – transgenic mice. Since their antigen (the model autoantigen) is not expressed in the donor mice these T cells are not negatively selected in the thymus. Importantly, the absence of antigen during T cell development ensures a completely naïve phenotype of the autoreactive T cells. This is crucial because it allowed, for the first time, investigation of the activation of T cells specific for a pancreatic self antigen *in vivo* (103). The other major advantage of adoptive transfer experiments is the ability to label the T cells with fluorescent dyes prior to injection, which allows them to be easily tracked *in vivo*. This technique can also reveal their activation and proliferation through analysis of cell division (104, 105). These innovations allowed many important aspects of T cell-mediated autoimmune responses to be addressed, including the site of activation of the autoreactive T cells, the influence of their numbers, their fate and the type of antigen-presenting cell responsible. Although parameters such as T-cell affinity and epitope spreading and recruitment of other autoreactive T cell clones are not taken into account, this approach has added considerably to our knowledge of immune-mediated destruction of islet β -cells by allowing us to dissect the actions of relevant components and their interactions which finally result in disease (102).

The approach described above has been particularly successful in elucidating the role and physiology of CD8 T effector cells. As compared to CD4 T helper cells, CD8 T cells are difficult to maintain *in vitro* as T cell lines, mainly because of their cytotoxicity. They quickly destroy APCs *in vitro*, and thus deprive themselves of antigenic survival signals. Furthermore, the MHC class I molecules, which are recognized by CD8 T cells, are expressed by nearly all murine cells, including T cells, so that CD8 T cells could potentially kill each other *in vitro*. These problems were overcome by generating naïve, and therefore unarmed CD8 T cells in transgenic donor mice. Such T cells became cytotoxic only after transfer into recipient mice expressing antigen. Transgenic CD8 T cells specific for certain MHC class I molecules were first generated (106). These T cells were introduced into transgenic mice expressing MHC I molecules under the influence of the rat insulin promoter (RIP) in pancreatic islet cells. This resulted in immune tolerance, unless the T cells were supplied with inflammatory mediators (107). These experiments demonstrated that autoreactive CD8 T cells can in principle destroy β -cells and cause diabetes (102) although their specificity for antigenic peptide was unclear. Therefore, they did not allow investigation of the processing of islet antigens. This limitation was overcome in the next generation of CD8 T cell transgenic systems. The first of these models, the RIP-mOVA model, expressed ovalbumin (OVA) as a model antigen in pancreatic islet cells. OVA-derived peptides could be detected with transgenic OVA-specific CD8 and CD4 T cells derived from OT-I and OT-II mice, respectively (OT-I and OT-II refer to the transgenic OVA-specific T cells, called OT-I and OT-II cells, that are produced by these transgenic mice). These experimental systems demonstrated clearly that both naïve CD4 and CD8 T cells with specificity for a pancreatic self antigen are activated by dendritic cells in the pancreatic lymph nodes, and not in the islets (108) (Fig 1). The DCs presumably took up β -cell antigens in the islets and carried them to the pancreatic lymph node for presentation to naïve T cells. In the case of CD8 T cells, these results demonstrated so-called cross-presentation, which denotes the presentation of extracellular antigens with MHC I molecules to CD8 T cells.

Since then, numerous observations of cross-presentation and cross-priming *in vivo* have been described, not only of transgenic self-antigens in models like the RIP-mOVA or the hemagglutinin system (109), but also of tumour (110) and viral antigens (111, 112). Thus, the basic mechanisms of antigen presentation and T-cell activation *in vivo* that were uncovered in transgenic systems have been verified for many 'natural' antigens. This is not surprising, because indirect presentation of antigen

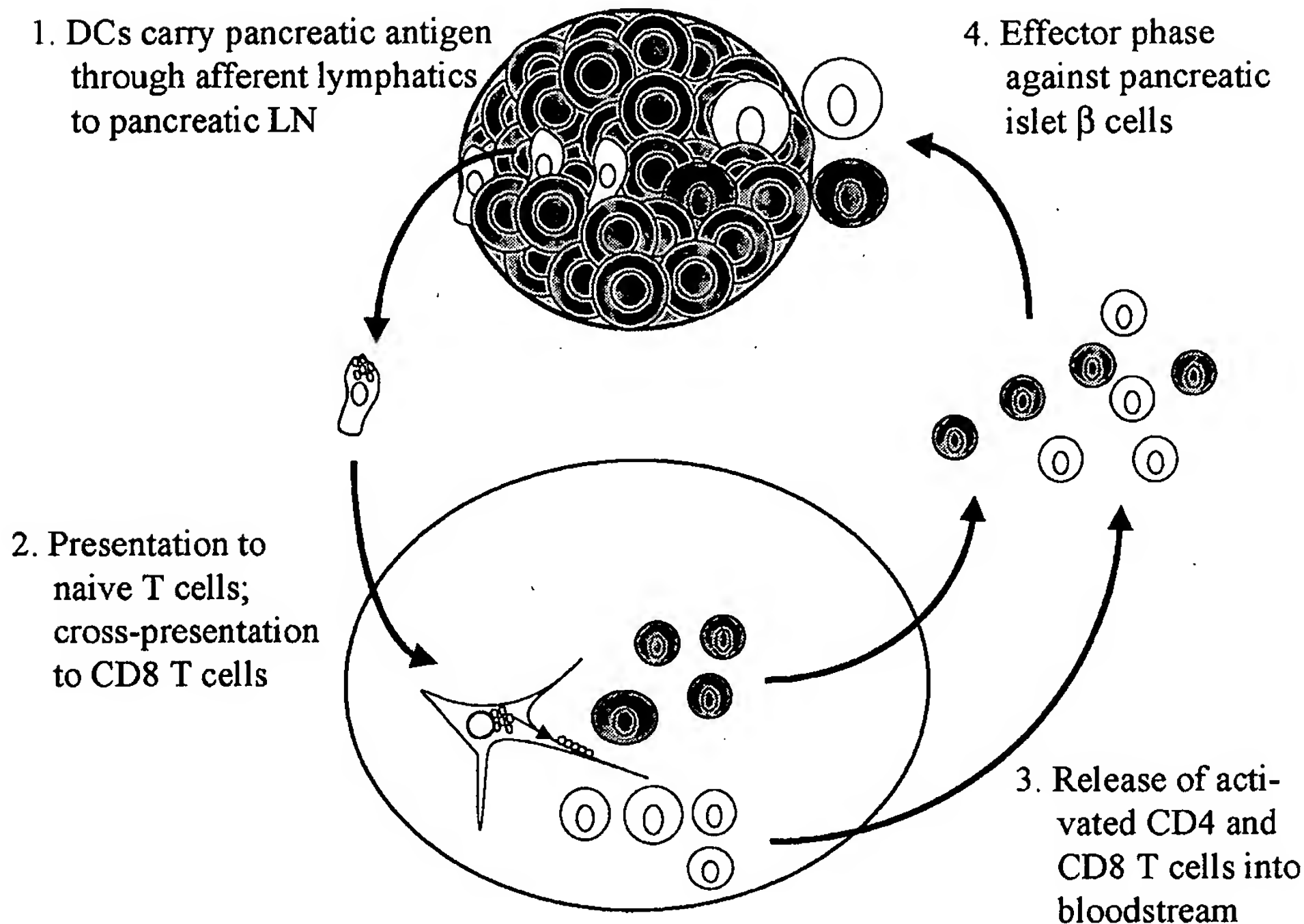


Figure 1. Antigen capture by dendritic cells in islets and their migration to the pancreatic lymph node for presentation of β -cell antigens to T lymphocytes. Antigen-presentation via both MHC II and MHC I pathways enables both CD4 and CD8 T cells to be activated. Activated T cells can enter non-lymphoid tissues such as pancreatic islets in search for antigen-expressing target cells (see text for details).

to T cells – both to CD4 and/or CD8 T cells – offers advantages to the immune system in the battle against infectious antigens. Only indirect antigen presentation (in the case of CD8 T cells, cross-presentation) allows an immune response to be mounted against viruses that functionally compromise, or simply avoid antigen-presenting cells (103).

These basic mechanisms of antigen presentation, which were uncovered in model systems such as the RIP-mOVA system, were found to be important also for the pathogenesis of diabetes in NOD mice, in which CD8 T cells have emerged as important mediators of disease (113–117). TCR-transgenic NOD mice have been generated, and in many of these, the CD8 T cells destroyed pancreatic islets, whereas the CD4 T cells played more or less relevant helper roles (115, 118, 119). In NOD mice, autoreactive CD4 T cells appear to play an important role in diabetogenesis, and also their activation occurs in the pancreatic lymph nodes (120). Interestingly, presentation of autoantigen to autoreactive CD4 and CD8 T cells is age-dependent. It did not occur in very young animals (120), suggesting that developmental changes in the pancreatic tissue cause the release of autoantigens to the pancreatic lymph node. In this respect, a

wave of physiological apoptotic β -cell death, which occurs in rodents at 14–17 days after birth (121), and in humans at birth (122), may be relevant. During such a wave, autoantigens are released from apoptotic islet cells and may activate autoreactive T cells with specificity for β -cell antigens (101, 123). Antigens released from apoptotic cells are taken up particularly well by dendritic cells (124–126) and normally, induce T cell tolerance (127). Why antigen-presentation results in autoimmunity in some individuals is not known, but inflammatory signals caused by accompanying infections (128), differences in the T cell repertoire, genetic susceptibility differences and dysregulated cellular death may be involved. Also, unphysiological β -cell death might cause the release of islet antigens in an immunogenic setting, and induce autoimmunity rather than tolerance (101). Tissue remodelling and replacement of β -cells occur also later. In the RIP-mOVA system, pancreatic tissue antigens were observed to be constantly shuttled to the draining lymph nodes of adult animals, where they were presented to autoreactive T cells (108, 120). These T cells indeed caused diabetes, but only when their precursor frequency was unphysiologically high (103). Physiological numbers of T cells were tolerized

by deletion before they could destroy all β -cells. Thus, cross-presentation of pancreatic self-antigen led to deletional CD8 T-cell tolerance, which was termed cross-tolerance (129). Cross-tolerance has also been shown in other transgenic systems, and its mechanisms have been elucidated (109). However, in some systems, most notably in those examining antigens derived from the lymphochoriomeningitis virus (LCMV), specific CD8 T cells ignored this self antigen (130) but were not deleted. One possible explanation is that various antigens are handled differently by the immune system. In this case, however, pathogens might easily evolve strategies to escape immune surveillance. An alternative explanation came from studies investigating the influence of antigen dose on cross-presentation. Only high antigen levels were cross-presented and induced cross-tolerance (131–133) while low dose self antigens were ignored, in which case the immune system must rely on ignorance to avoid autoimmunity. Thus, the level of antigen expression appears to determine the mechanism by which CD8 T-cell mediated autoimmunity is avoided.

Ignorance of self antigen allows autoreactive T cells to survive within the T cell repertoire. Such T cells could theoretically unleash their destructive potential if they were activated by other means, for example by pathogens that are similar in antigenic structure. This sword of Damocles has first been demonstrated in the LCMV system, where mice expressing determinants of the LCMV virus in pancreatic islets became diabetic after infection with the virus (134–135). Such a mechanism may explain why immune diseases are often observed after viral infections. Direct evidence for antigenic mimicry as a mechanism of diabetes induction, however, is scarce (136). In contrast to ignorance or to the induction of anergy or TCR downregulation in autoreactive T cells (137, 138) deletion of autoreactive T cells removes the threat of autoimmunity permanently by eliminating potentially harmful effectors. However, this mechanism can also fail, for example if the precursor frequency of the autoreactive CD8 T cells is too high, or when autoreactive CD4 T cells are present (103). Transgenic CD4 T cells specific for islet self-antigens appear to be less efficient as direct mediators of diabetes than CD8 T cells (139, 140). However, they act by delaying the deletion of CD8 effector T cells (139), by mediating their entrance into pancreatic islets (115) or by supporting their effector phase in the islets (107). But also the CD4 T cells themselves are subject to peripheral tolerance. CD4 T cells can be deleted (141) or functionally silenced (137). Also Th1 \rightarrow Th2 diversion may happen (142) and regulatory CD25⁺ CD4 T cells, that actively suppress immune responses (143) can be generated.

Molecular mechanisms regulating immune response to islet-antigens

As described above, antigens derived from islet- β -cells are under continuous surveillance by the immune system. Whether this antigen-presentation leads to expansion of islet-specific T lymphocytes and to the development of anti-islet immunity (Fig 1) – or to their silencing – is of crucial importance. Molecular mechanisms underlying this distinction are gradually becoming unravelled. Signalling via cytokine- and costimulatory receptors is of particular importance (144–147). For example, expression of the costimulatory ligand B7.1 or TNF- α on islets, and signalling between CD40L and CD40 can lead to the breakdown of the tolerant state to islet-antigens, as has been shown in NOD mice by antibody treatments and by expression of TNF- α or B7.1 on β -cells (148–150). The action of TNF- α depends, however, on the timing of its action and can also suppress β -cell destruction (149, 150).

The TNF- and TNF-receptor families include several members that may still turn out to be important in the regulation of islet-immunity. A good example is the demonstration that signalling through the TRANCE-RANK receptor-ligand pair is involved in the generation of CD25⁺ CD4⁺ regulatory T cells in the pancreatic lymph node (151). These cells were shown to be able to restrain diabetogenic CD8 effector T cells. The existence of regulatory T cells seems to be controlled by costimulatory molecules, because in the NOD mouse, lack of B7-2 or CD28 leads to acceleration of diabetes *via* impaired action of CD25⁺ CD4 regulatory T cells (152).

Functional silencing, that is 'anergy', is traditionally considered to result from TCR-ligation without simultaneous costimulation but may in fact require costimulation. Accordingly, CTLA-4 is an important negative regulator of the activity of T cells (153), and abrogation of its function leads to acceleration of diabetes in BDC2.5 NOD mice (153). Also regulatory cells including CD25⁺ CD4 cells (143) and Treg1 cells (154), are partly anergic themselves. Cytokines IL-10 and transforming growth factor (TGF) - β , produced by regulatory T cells, are important in peripheral tolerance via their inhibitory actions on dendritic cell activity and on T cell proliferation and differentiation (155, 156). Which role these regulatory cells play in human disease is not yet clear but is under intensive research.

Receptors that recognize pathogen-associated molecular patterns (PAMP) and are thus called pattern recognition receptors (PRR) are also important in the generation of immune responses (157). These receptors are expressed on cells of the innate immune system including dendritic cells and couple with intracellular signal transduction pathways that reg-

ulate gene expression. Binding of a ligand (i.e., a foreign pathogen) to such receptors can thus upregulate various functions of dendritic cells. Although it is quite unclear if PRR have any role in the pathophysiology of islet-specific autoimmunity, these receptors and their signalling pathways deserve attention when attempting to manipulate antigen presentation in pancreatic lymph node.

Consequently, costimulatory and/or death-receptors and cytokines expressed by the cells of our immune system form an intricate network of interacting receptors and ligands that influence the fate of islet-reactive T lymphocytes. This network contains a multitude of potential drug targets for attempts to restore tolerance to peripheral tissue antigens such as islet-antigens, some of which are depicted in Figure 2.

Selective modulation of immune response to islet-antigens

Restoration of tolerance to islet β -cells without compromising general immune function requires that immunomodulation selectively targets the immune response to β -cells. So far, tolerance has been induced experimentally by introducing the antigen in a con-

trolled fashion, i.e., without adjuvants, as a soluble protein and preferably *via* mucosal surfaces or the intravenous route (158, 159). As discussed above, the prospects of these manipulations as immune therapy to diabetes, however, remain very uncertain. Tolerance can also be induced in T cells specific for any given antigen by complexing antigenic peptides with MHC molecules to produce 'custom-made' T cell receptor ligands that are administered without adjuvants and thus in the absence of costimulatory signals (160, 161). This, however, requires detailed knowledge of the immunodominant epitopes of the antigen, and when effective, is likely to be strictly restricted to a narrow specificity of T cells that may not alone be responsible for disease pathogenesis. Thus, multiple T cell receptor ligands would need to be administered unless some of them would be able to induce active regulatory immunity (161).

Current knowledge of the cellular and molecular mechanisms underlying regulation of peripheral tolerance to islet-antigens suggests that new strategies of therapeutic manipulation of the detrimental islet-specific immune response await to be discovered. This would, however, require novel ways of introducing islet-antigens to the immune system in a way that would allow a tolerance-regulating mechanism to be

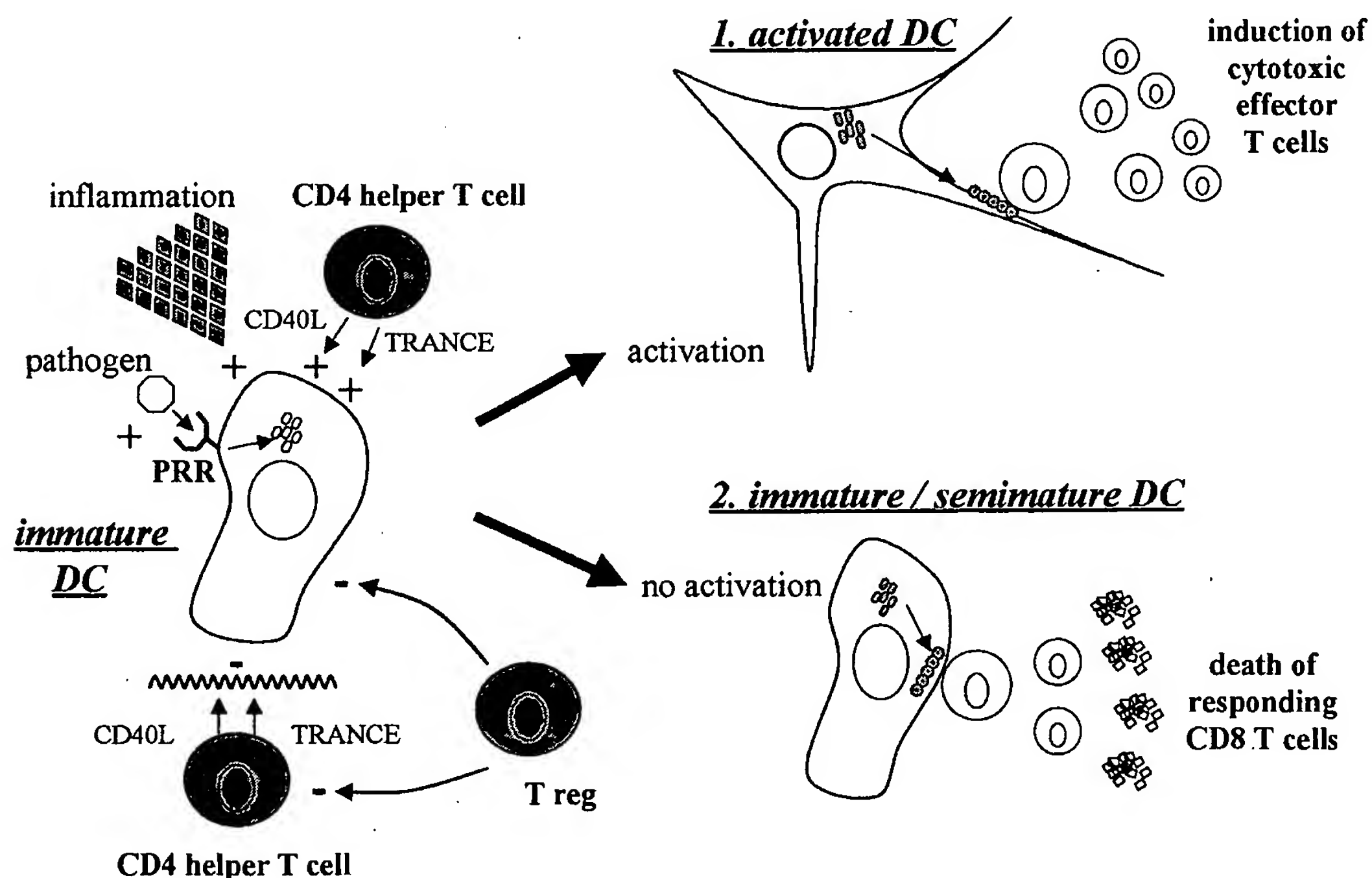


Figure 2. Factors that promote the activation of immature dendritic cell (DC) to become a potent antigen-presenting cell (1. activated DC) after endocytosis of antigen; and the role of regulatory T cells (Treg, e.g., CD25 + CD4 T cells) as opponents of the activation of immature DC (2. immature/semimature DC). PRR = pattern recognition receptor.

introduced simultaneously with the antigen in a strictly localized manner. Because T cells recognize antigen only when appropriately presented to them, this is possible only during T-cell interaction with an antigen-presenting cell or a target cell. In type 1 diabetes, the target cells (i.e., islet- β cells) are obviously beyond selective manipulation unless xenotransplantation of pig islets derived from transgenic pigs (whose islets would be made to express immunomodulatory agents) is considered relevant as a treatment option. Interaction with an APC which is presenting an autoantigen is the other antigen-specific interaction that a diabetogenic T cell commits itself to. Therefore, immunomodulation should probably be targeted here. If this APC cell were made to express an immunomodulatory agent, it could perhaps selectively target this agent to the T cell that is engaged with it *via* its antigen-specific receptor (162). This might re-direct the T cell to a less aggressive behaviour instead of its expansion and maturation into a clone of islet-destructive T cells. The immunomodulatory agent could be a death-inducing ligand such as FasL, TRAIL, TNF or PD-L1 (163–165). Experimental evidence suggests that expression of FasL on dendritic cells renders them tolerogenic (166, 167) although this may not always happen (168). In fact, we have observed that ligation of Fas during T cell contact with dendritic cells costimulates a fraction of the responding T cells (169) which could complicate its effects as a death-ligand. For other death-receptor ligands, data do not yet exist.

Alternatively, the immunomodulatory agent could be a cytokine such as IL-10 or TGF- β , because these cytokines regulate the costimulatory activity of the antigen-presenting cell and proliferation and polarity of the responding T cell as discussed above (155, 156). For therapeutic purposes, this type of targeted immunomodulation could be achieved by *in vitro*-treatment of autologous antigen-presenting cells with the immunomodulatory agent together with the antigen before re-injection, or by genetic modification to make these cells express the immunomodulatory agent themselves. Also, although B lymphocytes as well as monocytes can present antigen to T cells, dendritic cells would likely suit best to be used as modified antigen-presenting cells. This is because only dendritic cells are able to induce a response both in naïve and antigen-experienced T cells (170), and because B lymphocytes have an intrinsic property of directing islet-reactive T cells into diabetogenic behaviour at least in the NOD mouse (171, 172).

Modification of antigen-presentation from dendritic cells – a therapeutic option?

Dendritic cells can be propagated from peripheral

blood of humans with the aid of cytokines granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4 (173, 174). Such dendritic cells could be treated *in vitro* with an immunomodulatory agent or perhaps transfected with a gene construct to make them express the immunomodulatory agent themselves and pulsed with antigen before re-injection back into the same individual. *In vitro* cultured and antigen-pulsed dendritic cells from healthy individuals can, in fact, either induce a potent immune response or tolerance depending on their prior *in vitro*-treatment when re-injected (175, 176). The limitations of this approach would come from the amount of work and potential safety risks introduced by *in vitro* propagation of autologous cells. These limitations would not apply to the use of purified DNA as a 'vaccine' (177). The discovery that injecting 'naked' DNA can induce immunity was made a decade ago (178), and vaccination with 'naked' DNA represents a promising strategy for inducing cell-mediated immune responses (including cytotoxic CD8 T cells) (177, 178). Purified plasmid DNA encoding both an autoantigen and an immunomodulatory agent could therefore work as a 'vaccine' that might elicit a modified immune response resulting in tolerance instead of effective cell-mediated immunity. Unlike cells that need to be autologous, a vaccine consisting of recombinant DNA once validated could potentially be applied to individuals of a diverse genetic background (i.e., irrespective of MHC haplotypes or other disparate features). Thus, a 'vaccine' for people at risk of developing T1DM could perhaps become available on a large scale. The safety risks of introducing foreign recombinant DNA would probably be small if vectors that are able to incorporate into host genome (i.e., retroviral vectors) would not be applied.

In the NOD mouse, a few studies already exist in which DNA-plasmids encoding insulin, its B chain, GAD or hsp 60 either alone or together with a plasmid encoding a regulatory cytokine (e.g., IL-4) have been injected as a 'vaccine' to induce tolerance (47, 179–181). DNA injected as a 'vaccine' is taken up by cells in the tissue including dendritic cells which express the antigen and present it to T cells (182). In many cases this has resulted in regulatory immunity and protection from disease. This is noteworthy, given the capacity of DNA-vaccination to induce effective cytotoxic T-cell immunity in tumour and viral disease models (183, 184). Hence, the control of possible adverse immune reactions deserves careful attention when using this strategy. We believe that simultaneous expression of the autoantigen with an immunomodulatory agent could be a possible way to better control such adverse reactions and to target immunomodulation most effectively to pathogenic T cells. Intensive research is

yet needed in models such as the RIPmOVA -model and the NOD mouse to identify the most effective immunomodulatory molecules and the most relevant islet-autoantigens that these should be combined with.

A promising strategy to induce tolerance towards a defined antigen is to make a fusion protein consisting of the antigen and an antibody to an endocytosis receptor expressed on immature dendritic cells. Accordingly, the model antigen ovalbumin when conjugated to an antibody against the DEC-205 receptor, induced antigen presentation in dendritic cells that remained in an immature state (185). Presentation of antigen *via* immature dendritic cells rendered mice tolerant to subsequent challenge with the same antigen showing that targeting the antigen thoughtfully to the immune system may elicit antigen-specific tolerance.

We have discussed here the role of animal models in development of therapeutic strategies to induce immune tolerance to islet-autoantigens and envisioned some novel approaches to modify antigen-presentation from dendritic cells to induce tolerance (Fig 3). These strategies and approaches are currently under investigation in many laboratories including our own laboratories. Via innovative and careful work in animal models of islet-autoimmunity some of these approaches may be transformed into strategies that could be applied as specific immunotherapy to type 1 diabetes. Compared to treatment with general immunomodulatory or immunosuppressive agents, or other general, although certainly effective, treatment options like bone marrow reconstitution (186), tolerance-promoting presentation of autoantigens to the immune system would offer a worthwhile choice.

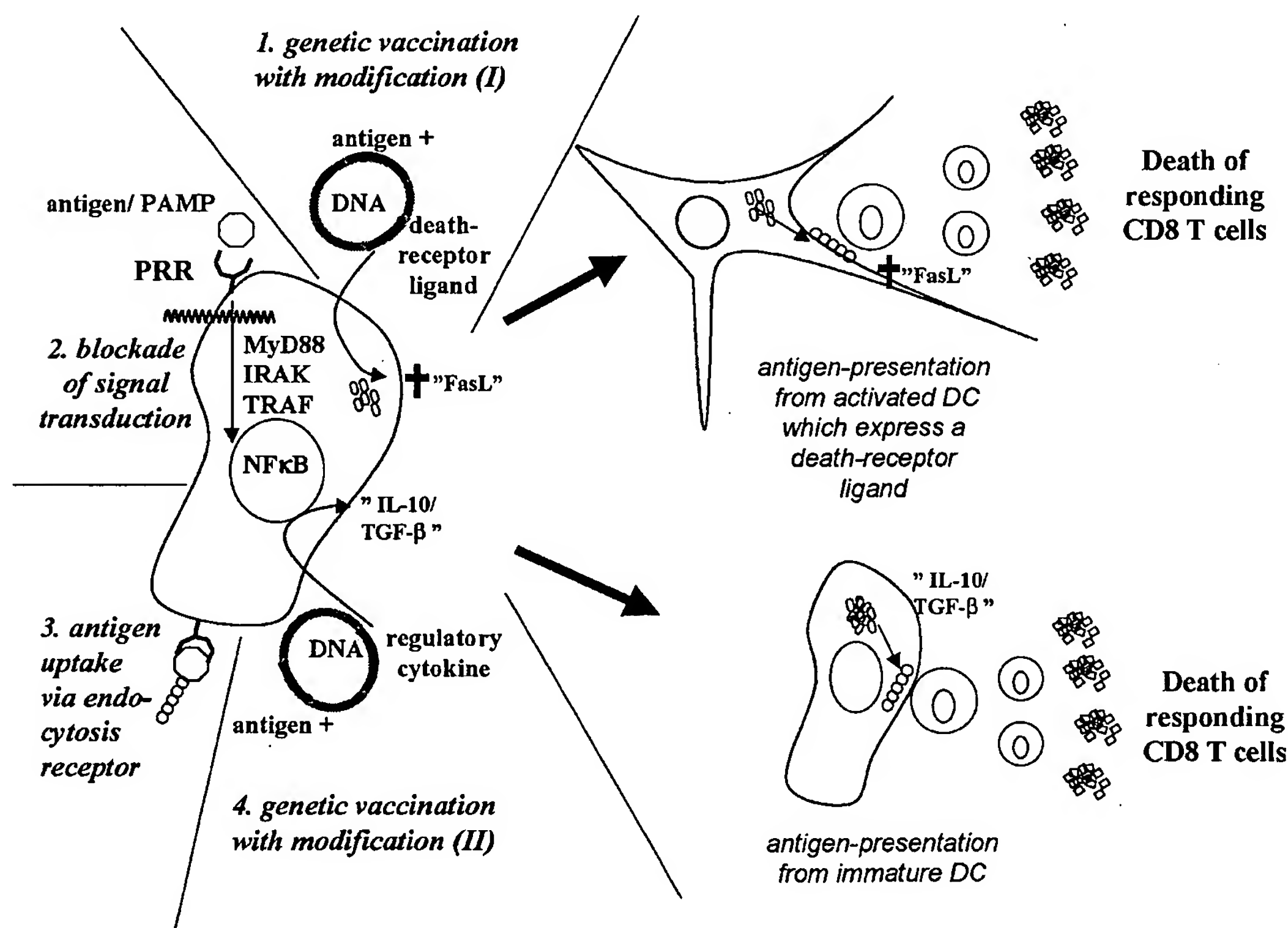


Figure 3. Four potential strategies for manipulating antigen-presentation in dendritic cells to induce tolerance. 1. Genetic (i.e. DNA) vaccination with a plasmid that encodes antigen and a death-receptor ligand (FasL, TRAIL, PD-L1/2); 2. Blockade of signal transduction from pathogen recognition receptor (PRR) by e.g., genetic modification of DC or antisense oligonucleotides; 3. Conjugation of antigen to a structure that binds to an endocytosis receptor (e.g., DEC-205) expressed on immature DC; 4. Genetic (i.e., DNA) vaccination with a plasmid that induces production of a regulatory cytokine (TGF- β , IL-10).

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